BACKGROUND. Impaired T-cell immunity in transplant recipients is associated with infection-related morbidity and mortality. We recently reported the successful use of adoptive T-cell therapy (ACT) against drug-resistant/recurrent cytomegalovirus in solid-organ transplant recipients.

METHODS. In the present study, we employed high-throughput T-cell receptor Vβ sequencing and T-cell functional profiling to delineate the impact of ACT on T-cell repertoire remodelling in the context of pre-therapy immunity and ACT products.

RESULTS. These analyses indicated that a clinical response was coincident with significant changes in the T-cell receptor Vβ landscape post-therapy. This restructuring was associated with the emergence of effector memory (EM) T cells in responding patients, while non-responders displayed dramatic pre-therapy T-cell expansions with minimal change following ACT. Furthermore, immune reconstitution included both adoptively transferred clonotypes and endogenous clonotypes not detected in the ACT products.

CONCLUSION. These observations demonstrate that immune control following ACT requires significant repertoire remodelling, which may be impaired in non-responders due to the pre-existing immune environment. Immunological interventions that can modulate this environment may improve clinical outcomes.
T cell repertoire remodelling following post-transplant T cell therapy coincides with clinical response

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Running title: TRBV reconstitution following CMV-specific adoptive immunotherapy

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Abstract

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**Introduction**

In contrast to acute viral infections that are cleared from the body within a few weeks, human cytomegalovirus (CMV) establishes lifelong latency within the host following acute infection (1). CMV is ubiquitous worldwide, infecting most of the population in early childhood and adolescence (2). In the vast majority of individuals, infection is unrecognised and constant viral reactivation is controlled by CD4+ and CD8+ T lymphocytes, which are maintained at high frequencies in the periphery and patrol sites of latency (3, 4). These memory T-cell populations display unique functional characteristics, including immediate effector function upon recognition of cognate antigen (5, 6). The T-cell landscape in individuals asymptomatically infected with CMV is dominated by the stable oligoclonal expansion of antigen-specific T cells (3, 7). CMV-specific T cells therefore function to maintain equilibrium with reactivating virus for the entire human lifespan in most individuals. However, this equilibrium is disrupted in transplant recipients who, due to an intensive immunosuppressive regimen, may be unable to develop normal immunological memory (8, 9). This can manifest as chronic viral disease, leading to end-organ disease and graft loss (9). There is now compelling evidence in a number of solid-organ transplant (SOT) settings, including lung, kidney, and heart, that CMV reactivation and disease is associated with the failure to establish stable immunological memory against CMV (8, 10-12).

Despite clear evidence of the importance of efficient immunological surveillance in the prevention of CMV disease in SOT patients, current prophylactic and therapeutic interventions for CMV are dependent upon the administration of anti-viral therapy, rather than immunological reconstitution (13). Whilst this is an efficient means to prevent viral reactivation, the emergence of drug resistance or intolerance results in viral reactivation that is often treated with secondary anti-viral therapy such as foscarinet, which can lead to
significant adverse effects that most patients do not tolerate long-term (13, 14). CMV-specific immune reconstitution offers an alternative approach to prevent ongoing viral complications without the need for long-term anti-viral therapy. We have developed an adoptive cellular therapy (ACT) approach to restore immunological control in patients with CMV complications. This approach is based upon a 2-week in vitro expansion of autologous CMV-specific T cells using a customised peptide pool containing 30 HLA class I and II restricted CMV-encoded epitopes. Following two case reports of successful treatment of lung transplant recipients with autologous CMV-specific ACT (15, 16), we recently completed an open-label phase I clinical study treating SOT recipients (lung, kidney, and heart) with autologous CMV-specific ACT (17). In total, we have now treated 13 patients with autologous CMV-specific ACT and met our primary objective of demonstrating safety, with no evidence of severe adverse events or graft-related events associated with T-cell infusion. In our clinical studies, 11 of 13 patients showed evidence of a clinical response to therapy, predominantly characterised by a reduction in viral load in the peripheral blood or an improvement in organ-specific disease symptoms. The remaining two patients showed no benefit from ACT. In this follow-up study, we sought to explore the impact ACT has upon the peripheral T-cell repertoire and explore potential correlates of improved clinical outcome in responding patients.
Results

Clinical responses to CMV-specific ACT are associated with changes in the T-cell receptor Vβ (TRBV) repertoire

A summary of clinical data from the SOT recipients included in the current study is provided in Table 1 and a schematic representation of the current study strategy is provided in Figure 1. Ten participants from our previously published clinical trial (17) were selected for the current analysis based upon the availability of peripheral blood mononuclear cells (PBMC) following ACT. These patients received CMV-specific T cells (median: $1.92 \times 10^8$, range: $4.2 \times 10^7$ to $2.45 \times 10^8$) in 2–6 intravenous infusions (Table 1). CMV-specific T cells expanded from all SOT recipients showed strong virus-specific reactivity (median: 57.1%, range 22.9% to 77.2%) and the majority of cell therapy products were dominated by CD8+ T cells (Table 1), with no detectable virus-specific CD4+ T cells. Eight of the ten patients, characterised as responders, displayed evidence of improvement in CMV clinical symptoms, demonstrated by a reduction in viremia, end-organ disease resolution and/or cessation of anti-viral therapy following ACT (Table 1). Of the two non-responding patients, one died of multi-organ failure with no improvements in clinical symptoms and the other displayed ongoing elevated viremia. We first aimed to delineate the impact of ACT on the SOT recipients’ global CD8+ T-cell repertoire, functional CD8+ T-cell landscape and clinical response. CD8+ T cells were sorted from peripheral blood samples collected either prior to the commencement of T cell therapy (pre-therapy), 4–8 weeks after the completion of therapy (post-short term; post-ST), or 20–29 weeks after the completion of therapy (post-long term; post-LT), and TRBV deep sequencing analysis performed. A summary of the TRBV analysis is provided in Supplemental Table 1. Prior to the commencement of therapy, patients displayed disparate TRBV clonotypic compositions. Despite ongoing complications with CMV, some patients displayed dramatic
oligoclonal expansions associated with CMV exposure, whereas others showed very little repertoire bias (Fig. 2A). The TRBV complementarity-determining region 3 (CDR3) landscape in these patients, exemplified by patient PAH08, was dominated by low-frequency clonotypes. This lack of pre-therapy clonal expansion was associated with a more dramatic change in the TRBV landscape post-ACT. Patients PAH08, PAH09, PCH02 and PCH04 displayed the greatest restructuring of their CD8 TRBV repertoire following ACT, characterised by a reduction in diversity and a dramatic increase in clonality (Fig. 2B). The fold change in clonality after therapy inversely correlated with the pre-therapy clonality (Fig. 2C), suggesting that patients with a less biased repertoire were more receptive to ACT-mediated changes in the TRBV landscape.

Changes in clonotypic composition following ACT are associated with significant clonotypic expansion

To further delineate the impact of ACT on T-cell repertoire, we used a two-sided binomial test to determine the number of clonotypes in each patient that displayed significant changes following ACT. All SOT recipients whose clinical symptoms resolved following ACT showed significant clonotypic expansion post-ACT (Fig. 3A and Supplemental Fig. 1). However, only one expanded clonotype was detected from the non-responding patient, RAH01 (Fig. 3B). Similarly, patient PCH03 showed no expanded clonotypes following ACT. While this observation may reflect the reduced sample coverage in these individuals due to poor CD8+ T cell yield from PBMC, it does suggest an association between outcome and clonotypic expansion post-ACT. While the most dramatic changes were evident in PAH08, in whom we observed a significant increase in 334 different clonotypes post-therapy, other responders,
particularly PCH06, who did not display a change in clonality post-therapy, showed clear evidence of significant clonal expansion. This included the emergence of clonotypes that were undetectable prior to ACT, indicative of the emergence of novel clonotypes that likely played a role in immune control of CMV infection and/or disease following ACT. We also noted that a number of CMV-specific clonotypes were present in these expanded populations, in addition to other CMV-specific clonotypes that did not display significant expansion post-therapy (Table 2). These significantly expanded clonotypes constituted >15% of the productive TRBV repertoire in all responding patients long-term post-ACT (Fig. 3C). Most patients displayed bias in their TRBV usage, with significant changes detected in multiple TRBV families, which demonstrates the diversity of changes post-ACT.

To examine the relationship between the expanded clonotypes, we next performed grouping of lymphocyte interactions by paratope hotspots (GLIPH) analysis to determine the immunodominant CDR3 motifs in SOT recipients following ACT (18). In brief, GLIPH analysis considers all possible amino acid sequences (K-mers) of length 4, in a population of CDR3s and reports K-mers that are statistically over-represented (read motif) when compared with a naïve repertoire database. In most responding patients, we were able to identify motifs within the CDR3 sequences that expanded post-ACT (Supplemental Table 2). Representative analyses displaying motifs of >0.1% frequency either pre- or post-ACT in three responders are shown in Fig. 2D. These analyses suggest that changes in the CDR3 landscape, associated with a dramatic increase in clonality and/or the significant expansion of virus-specific clonotypes, were associated with an improved outcome in treated patients.
ACT products from different patients display unique specificity and structural composition

We next explored the association between T-cell clonotypes within each ACT product and changes in TRBV repertoire landscape following ACT. The ACT products generated for most patients contained multiple CMV specificities restricted through one or more HLA alleles (Fig. 4A). CMV-specific T cells enriched from ACT products using a cytokine capture protocol (Supplemental Fig. 2 and Supplemental Table 3) showed a range of diversities with predominantly unique clonotypic architecture. Circos plots from the individual patients demonstrate little overlap in the paring of TRBV and TRBJ genes, indicative of unique T-cell receptor usages in the different ACT products (Fig. 4B).

To further assess the relationships between the clonotypic composition of ACT products from different patients, we compared TRBV and CDR3 usage. A small number of ACT products displayed overlap in TRBV usage (Fig. 5A) and CDR3 sequences, and this was most evident in patients PAH09 and PCH04, who shared 23 clonotypes and had an immunodominant response to the HLA-C*06:02-restricted TRA epitope (Fig. 5B). However, very little overlap in clonotypic composition was seen between most patients’ ACT products, despite the presence of CMV-specific T cells recognising the same epitopes. We also saw no clear differences in TRBV usage and clonotypic composition when comparing the ACT products of responding and non-responding patients.

Post-ACT immune reconstitution is associated with the clonotypes present in the ACT product

To examine the reconstitution of clonotypes associated with the ACT products, we identified the top 20 dominant clonotypes in each product. These are represented in Figure 6A as a
proportion of the total productive rearrangements in each product. To assess changes in the frequency of these clonotypes, we tracked them over time following ACT (Fig. 6B). Global clonotypic analysis revealed that an increase in the frequency of cell therapy-associated clonotypes was most evident in patients with a more diverse repertoire pre-therapy, particularly patients PAH08, PCH02 and PCH04. However, changes in frequency did not necessarily correlate with the pre-therapy clonotypic diversity, nor with immunodominance in the ACT product. For patient PAH08, two immunodominant clonotypes that were present in pre-ACT PBMC and detected at a high frequency in their ACT product declined following treatment, in contrast to the majority of their other clonotypes. Similarly, patient PCH06’s immunodominant HLA-A2-restricted NLV epitope-specific clonotype (see Table 1) declined post-therapy, while a significant expansion in other clonotypes specific for this epitope was seen.

Clonotypic expansion following ACT is not restricted to the clonotypes present in the ACT products

We next assessed the relationship between the clonotypic composition of the ACT product generated for each patient and the significantly expanded clonotypes detected post-therapy. We determined whether or not the significantly expanded clonotypes outlined in Figure 2 were detectable in the ACT product or PBMC collected prior to the commencement of ACT, and what contribution these clonotypes made to the TRBV landscape post-therapy. Due to the low number of significant clonotypic expansions in the products of the two non-responders (one for patient RAH01 and none for patient PCH03), this analysis included only the eight responders. The majority of SOT recipients who showed clinical improvement following ACT demonstrated a significant increase in the frequency of ACT product-associated
clonotypes post-therapy (Fig. 7). In these patients, we also observed the emergence of clonotypes that were undetectable pre-therapy or in their ACT product. These observations indicate that immune reconstitution following CMV-specific ACT not only includes adoptively transferred clonotypes, but also impacts upon anti-viral immunity by promoting the expansion of endogenous clonotypes.

**Immune reconstitution is associated with the expansion of EM T-cell populations post-therapy**

To assess the relationship between changes in the CDR3 landscape with changes in CMV-specific T-cell immunity, we analysed the frequency of MHC multimer-specific T cells in the peripheral blood. This analysis was performed on seven patients for whom MHC multimers were available. PBMC from all SOT recipients contained CMV-specific T cells prior to immunotherapy, with the frequency of HLA-A2-restricted NLV epitope-specific T cells exceeding 23% in PCH06, which correlated with the presence of the immunodominant NLV epitope-specific CDR3 sequence in the TRBV analysis (Fig. 8A). While frequencies of CMV-specific T cells did not change dramatically post-ACT, the emergence of HLA-A*01:01-restricted VTE epitope-specific T cells in participants PCH02 and PAH08, and HLA-B*08:01-restricted ELK epitope-specific T cells in PAH08 was observed. These changes were associated with an increase in the presence of cytokine-producing CMV-specific T cells. An increase in the frequency of HLA-C*06:02-restricted TRA epitope-specific T cells was observed in the PBMC of participant PCH04, and was associated with an increase in the frequency of IFN-γ-producing antigen-specific T cells following ACT. While the HLA-A*02:01-restricted NLV epitope-specific T-cell frequency in PCH06 declined, this was not associated with a decrease
in the frequency of functional T cells, suggesting the new NLV epitope-specific clonotypes that emerged post-ACT constitute the functional component of this response.

To determine the phenotypic profile of the CMV-specific T cells pre- and post-ACT we assessed the co-expression of CCR7, CD45RA, CD28, CD27, CD57, and CD95 in combination with MHC multimers. For each participant, samples were concatenated and tSNE analysis was performed. This identified six predominant populations within global CD8+ T cells, characterised by differential surface marker expression (Supplemental Fig. 3). The emerging CMV-specific T-cell populations, represented by HLA-A*01:01-restricted VTE epitope-specific T cells in patients PAH08 and PCH02, displayed an EM3 phenotype that was associated with high levels of CD57 expression and low expression of CD27 and CD28 (Fig. 8B). This EM phenotype was also present post-ACT in the HLA-B*0801-restricted ELK epitope-specific T cell from PAH08 (data not shown). In contrast, the HLA-C*06:02-restricted TRA epitope-specific T cells from PAH09 and PCH04 had a mixed phenotype, with both central memory (CM) and EM populations present before and after immunotherapy (Fig. 8B). Similar observations of a mixed CM/EM phenotype were also present in HLA-A*02:01-restricted NLV epitope-specific T cells in other patients (data not shown). These phenotypic differences are consistent with our previous observations in healthy virus carriers (19).

**Immune reconstitution is associated with global T-cell phenotypic changes post-therapy**

To more broadly explore the global T-cell phenotype in trial participants before and after ACT, we used tSNE analysis to define memory populations in the global CD8+ T-cell population. This analysis included eight of the patients for whom post-LT samples were available, excluding patients PCH01 and PCH03. To compare this analysis to functional T-cell reconstitution in
these patients, we assessed their CMV-specific polyfunctional cytokine profile through the expression of IFN-γ, TNF and IL-2, and degranulation via CD107a surface expression. Polyfunctional cells were defined as those T cells capable of generating two or more functions (typically IFN-γ⁺CD107a⁺TNF⁺). Monofunctional cells were those capable of producing only one function (typically IFN-γ⁺). Supporting our observations from CDR3 analysis, T cells from the majority of the responding patients displayed proportional changes in memory profile following ACT, characterised by an increase in the proportion of EM T cells (Fig. 9A). The changes were particularly evident in PCH02, PCH04, PAH08 and PAH09, whose CD8⁺ T-cell populations were dominated by naïve T cells prior to ACT. These dramatic changes in clonotypic composition were associated with an expansion of EM3 CD8⁺ T cells in patients PCH02, PCH04 and PAH08, and EM1, 2 and 3 T cells in PAH09. These patients also demonstrated an increase in the number of CMV-specific polyfunctional T cells following ACT (Fig. 9B). Similarly, although a high proportion of EM3 cells was observed in CD8⁺ T cells from PCH06 and PAH06 prior to therapy, post-ACT samples also showed an increase in the proportion of EM cells, while a high frequency of EM T cells was maintained in PCH05. These patients maintained a similar frequency of polyfunctional T cells following ACT. In contrast, participant RAH01 displayed a high proportion of both CM and EM populations pre-therapy and negligible naïve T cells (Fig. 9). Consistent with our observation of very little change in the TRBV landscape in this patient and no evidence of virus-specific T-cell reconstitution, this cellular phenotype did not change post-ACT. This patient also displayed very few polyfunctional CD8⁺ T cells following ACT.

Finally, to assess the potential impact the inflammatory milieu could be having upon T cell expansion following ACT, we used a Human Cytokine/Chemokine 65-Plex Panel to assess
expression in plasma from the ten ACT recipients prior to infusion. While we saw no clear patterns of differential cytokine or chemokine expression in responding patients that could reflect improved reconstitution following ACT, we noted elevated levels of G-CSF, IL-10 and IL-28A in plasma from one of our non-responders, RAH01, prior to infusion (Supplemental Fig. 4). In both non-responders we also saw reduced expression of CCL15, a chemokine shown to promote recruitment into the lung. While these observations provide some indications of the potential impact the inflammatory milieu has upon T cell reconstitution following ACT, they need to be further explored in a larger cohort or in a controlled pre-clinical model.
Discussion

In this study, we sought to explore the impact of adoptive anti-viral T-cell immunotherapy on the peripheral T-cell landscape in SOT recipients who had developed CMV-associated complications due to drug resistance or disseminated end-organ disease. Surprisingly, we noted significant diversity in the composition of the CD8+ T-cell repertoire in responding patients prior to ACT; some patients displayed typical clonal expansions associated with persistent CMV infection, whilst others showed little bias in their repertoire. In the majority of trial participants who responded to ACT, changes in peripheral T-cell repertoire were associated with dramatic restructuring of the T-cell landscape and/or the expansion of virus-specific clonotypes following ACT. In contrast, a biased peripheral T-cell repertoire that was not altered by ACT in non-responding recipients suggests that the restructuring of the TRBV landscape following ACT was critical for the clinical improvements seen in the responding patients.

Persistent CMV infection has a dramatic impact on the T-cell compartment in humans. This impact was initially demonstrated through the identification in seropositive individuals of expanded EM T-cell populations (20), high frequencies of CMV-specific T cells, and the associated oligoclonal TRBV phenotype (3). More recent observations have shown the dramatic impact of CMV on global CD8+ TRBV architecture, characterised by a CMV-specific signature that can be used to differentiate exposed and non-exposed individuals (21). CMV reactivation and viral control in haematopoietic transplant recipients was recently associated with a rapid dramatic restructuring of both the EM and naïve T-cell repertoire (22). In contrast, we have previously reported that oligoclonal expansion can also be associated with poor viral control (23), indicative of the importance of T-cell programming and functionality in promoting viral control (24). In the SOT setting, the establishment and maintenance of
functional CMV-specific immunity has long been established via the analysis of cytokine production (9, 25, 26), which has now been standardised for use in a number of diagnostic assays (27-29). Other recent observations have also demonstrated that efficient immunity against CMV is not just dependent upon the presence of a high frequency of CMV-specific T cells, but also their correct functional programming (30, 31). In the current study, we observed evidence for dramatic repertoire skewing prior to ACT despite underlying defects in viral control, further emphasising the importance of functionality in these populations. While patients with little repertoire skewing appeared most responsive to ACT, it was evident that dramatic changes in the clonotypic composition could also occur in patients with existing oligoclonal expansions. Interestingly, clonotypic changes were not necessarily associated with pre-existing immunodominance hierarchies or immunodominance hierarchies in the ACT products. This suggests that other properties of the T cells, such as phenotype and antigenic target, likely influence post-therapy expansion.

While our preliminary observations demonstrate an association between the control of CMV disease-related symptoms and the restructuring of the TRBV landscape following ACT, the mechanisms that prevent efficient T-cell mediated control in non-responsive patients remain to be elucidated. However, it was clear that both non-responding patients had pre-existing clonal expansions. Although speculative, based on only two patients, it is plausible that underlying immunological defects associated with these dramatic clonotypic expansions may limit the ability of adoptively transferred T cells to expand and control virally infected cells. The removal of these populations through lymphodepletion could provide one strategy to improve the reconstitution of transferred T cells. This type of approach is successful in other settings of ACT, particularly in cancer patients (32, 33); however, it would also have the potential to deplete effector T-cell populations that play a role in viral control.
Lymphodepletion prior to ACT in SOT recipients would therefore need to preferentially target these oligoclonal EM T-cell populations. However, this type of approach requires intensive treatment and would require larger cohort studies to better define the impact of the pre-ACT immunological environment on post-ACT immune reconstitution. An alternate approach would be to administer ACT prior to the dramatic changes in the TRBV landscape that are evident in some patients in our cohort. Although we cannot definitively say that immune control in patient PAH08 was mediated by the administered T cells, this patient provides the strongest evidence that a lack of pre-therapy clonal expansions is associated with post-therapy changes in the TRBV landscape. At-risk patients could be monitored for both viremia and CMV-specific immunity following viral reactivation, to allow intervention to occur during earlier stages of viral reactivation (9, 10). Other potential avenues to improve immune reconstitution following ACT could incorporate modulation of the cytokine environment prior to or during ACT. Although not definitive, our preliminary observations suggested some potential differences in the inflammatory milieu prior to cell therapy. Other confounding factors, including the ongoing co-administration of anti-rejection and anti-viral medications, also likely have an impact on the efficacy of ACT. However, due to the limited size of the current cohort, it is not possible to assess this impact.

Another potential limitation on the success of autologous ACT for SOT patients is the quality of the cellular product. While we were unable to define clear differences in the ACT products of responders and non-responders, it was evident that one of the non-responders contained a near-monoclonal T-cell population dominated by a single TRBV sequence. While this was also observed in some of the responding patients, T-cell receptor diversity is known to play an important role in viral control (34, 35). Other unexplored functional defects are also likely present in ACT products generated from heavily immunosuppressed patients, which
may impact the efficacy of these products. Another approach to treating viral complications, which has been well studied in haematopoietic stem cell transplant recipients, is the use of HLA-matched allogeneic virus-specific T cells (36, 37). This approach utilises healthy donors as a source of PBMC for the manufacture of T cells. It has the potential to provide cells selected to have optimal functionality, broader specificity and increased clonotypic diversity from donors not exposed to heavily immunosuppressive drugs. It also allows for rapid administration of ACT from banked cryopreserved cells.

Cellular immunotherapy is emerging as a powerful strategy to restore immunological control against CMV and other common pathogens affecting SOT recipients and other immunosuppressed patients. Understanding the immunological environment pre-therapy, and post-therapy changes associated with immune control will provide a platform to better enhance the future clinical use of these approaches.
Methods

Patient treatment

The single-arm open-label phase I adoptive immunotherapy study, including the preparation of the ACT, has been previously described (17). Briefly, autologous CMV-specific T cells were generated from PBMC using a custom-designed peptide pool that included 30 defined CMV-encoded epitopes. Patients were administered up to six doses of T cells at fortnightly intervals and monitored for 28 weeks after the completion of therapy.

Isolation and deep sequencing of CD8⁺ T cells

To isolate CD8⁺ T cells, PBMC were incubated with anti-CD8-PerCP-Cy5.5 (clone RPA-T8, eBioscience, San Diego, USA), anti-CD4-FITC (clone RPA-T4, BD Biosciences, New Jersey, USA), anti-CD3-APC (clone SK7, BD Biosciences), and LIVE/DEAD fixable near-IR dead cell stain (ThermoFischer, Waltham, USA). CD3⁺CD8⁺CD4⁻ viable lymphocytes were sorted using a BD FACSAria III. DNA was isolated from the sorted cells using Qiagen DNeasy Kit (Qiagen, Hilden, Germany). To isolate IFN-γ-producing CD8⁺ T cells, T cells generated for ACT (ACT product) were stimulated with the CMV peptide pool and cultured for 4 hours. IFN-γ was captured on cells using an IFN-γ secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany) with a PE-conjugated anti-IFN-γ detection antibody. Cells were co-stained with anti-CD8-PerCP-Cy5.5, anti-CD4-FITC, anti-CD3-APC, and LIVE/DEAD fixable near-IR. CD3⁺CD8⁺CD4⁻IFN-γ⁺ viable cells were sorted using a BD FACSAria III (Becton Dickinson), and DNA isolated using the Qiagen DNeasy Kit. The isolated DNA was then sent to Adaptive Biotechnologies® (Seattle, WA, USA) for TRBV deep sequencing analysis using the immunoSEQ platform.
Analysis of TRBV sequencing

Library preparation and sequencing: Sample data were generated using the immunoSEQ assay. The somatically rearranged human TRBV CDR3 was amplified from genomic DNA using a two-step, amplification bias-controlled multiplex PCR approach (38, 39). Briefly, the first PCR consists of forward and reverse amplification primers specific for every V and J gene segment, and amplifies the hypervariable CDR3 of the immune receptor locus. The second PCR adds a proprietary barcode sequence and Illumina adapter sequences. CDR3 libraries were sequenced on an Illumina Nextseq 500 instrument according to the manufacturer’s instructions.

Data analysis: Raw Illumina sequence reads were demultiplexed according to Adaptive’s proprietary barcode sequences. Demultiplexed reads were then further processed to: remove adapter and primer sequences; identify and correct for technical errors introduced through PCR and sequencing; and remove primer dimer, germline and other contaminant sequences. The data were filtered and clustered using both the relative frequency ratio between similar clones and a modified nearest-neighbour algorithm, to merge closely related sequences. The resulting sequences were sufficient to allow annotation of the V(N)D(N)J genes constituting each unique CDR3 and the translation of the encoded CDR3 amino acid sequence. V, D and J gene definitions were based on annotation in accordance with the IMGT database (www.imgt.org). The set of observed biological TRBV CDR3 sequences was normalised to correct for residual multiplex PCR amplification bias and quantified against a set of synthetic TRBV CDR3 sequence analogues (38). Initial alignment and bioinformatics was performed using the ImmunoSEQ Analyzer platform.
Additional analysis of TRBV was performed using VDJtools (version 1.1.10) (40) and the R package “tcR” (version 2.2.1.11) (41) using default settings, unless otherwise stated. GLIPH analysis was done using default settings in version 1.0 (18).

TCR sequencing data have been deposited in Adaptive Biotechnologies’ ImmuneACCESS database (doi:10.21417/CS2019JCI; https://clients.adaptivebiotech.com/pub/smith-2019-JCI)

**Phenotypic characterisation of PBMC**

PBMC were incubated with APC-labelled MHC class I multimers specific for the HLA-A*01:01-restricted epitope VTEHDTTLV (VTE), the HLA-A*02:01-restricted epitope NLVPMVATV (NLV), the HLA-B*07:02-restricted epitopes TPRVTGGGAM (TPR) and RPHERNFGTVL (RPH), the HLA-B*08:01-restricted epitope ELKRKMIYM (ELK), supplied by Immudex (Copenhagen, Denmark); or the HLA-C*06:02-restricted epitope TRATKMQVI (TRA), kindly supplied by the NIH tetramer facility (Emory University, Atlanta, USA). Cells were then incubated with anti-CD4-PE-Cy7 (Clone RPA-T4, BD Biosciences), anti-CD8-PerCP-Cy5.5 (Clone RPA-T8, eBiosciences), anti-CD19-PE-Cy5 (Clone HIB19, BD Biosciences), anti-CD27-PE (Clone L128, BD Biosciences), anti-CD28-BV480 (Clone CD28.2, BD Biosciences), anti-CD45RA-FITC (Clone HI100, BD Biosciences), anti-CCR7-AF700 (Clone 150503, BD Biosciences), anti-CD95-BV421 (Clone DX2, BD Biosciences), anti-CD57-BV605 (Clone NK-1, BD Biosciences), and LIVE/DEAD fixable near-IR (Life Technologies). Cells were acquired using a BD LSRFortessa with FACSDiva software (BD Biosciences) and post-acquisition analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR). To define phenotypically distinct T-cell populations, t-distributed stochastic neighbour embedding (tSNE) analysis was performed using FlowJo software. tSNE analysis was performed on each concatenated sample from each patient separately, and T-cell populations were characterised based on surface marker expression (Supplemental Fig. 3).
**Intracellular cytokine analysis of PBMC**

To characterise the ACT products and PBMC isolated from follow-up blood samples, cells were stimulated with CMV peptide epitopes and assessed for the expression of IFN-γ (Clone B27, BD Biosciences), TNF (Clone Mab11, BD Biosciences), CD107a (Clone H4A3, BD Biosciences) and IL-2 (Clone MQ1-17H12, BD Biosciences) as previously described (17). Cells were acquired using a BD LSRFortessa with FACSDiva software. Post-acquisition analysis was performed using FlowJo software. See Supplemental Table 3 for a list of CMV epitopes used in this study.

**Analysis of plasma cytokine and chemokine profile**

To explore the inflammatory milieu in patients prior to the commencement of adoptive cell therapy, plasma was harvested from peripheral blood and stored at -70°C prior to use. Plasma was then shipped to Eve Technologies (Calgary, Canada) and a 65-plex human cytokine/chemokine multiplex immunoassay was performed. The multiplex immunoassay was performed using a Milliplex assay (MerckMillipore, Burlington, USA) and analysed using a BioPlex200 (Bio-Rad, Hercules, USA). Cytokine and chemokine concentrations were calculated using standard curves for each target molecule.

**Statistical analysis**

Enrichment of productive TRBV CDR3s was assessed using the immunoSEQ® analyser. Only clonotypes with a minimum of five template reads were included in the analysis. Significance was determined using a two-sided binomial test with Benjamini–Hochberg multiple comparisons’ correction, where α = 0.01. To assess the correlation between fold change in clonality and pre-therapy clonality a two-tailed non-parametric Spearman’s correlation was used.
Study approval

The study was performed according to the principles of the Declaration of Helsinki. It was approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee, The Prince Charles Hospital Human Research Ethics Committee and the Royal Adelaide Hospital Research Ethics Committee, and registered under the Australian New Zealand Clinical Trial Registry (ACTRN12613000981729).
**Author Contribution:** C.S., M.A.N, K.K.M, S.C., R.F., D.C. and R.K. designed the study. C.S., D.C., L.B., S.R., P.C., M.S. and L.L.T. performed experimental work and analysis of the data. All authors contributed to the writing of the manuscript.

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References


Figure 1: Schematic representation of the study. Autologous CMV-specific T cells were expanded in vitro for 2 weeks. Patients received up to six doses of autologous CMV-specific T cells over a period of 14 weeks. Patients were monitored for up to 29 weeks following the final infusion. PBMC samples pre-infusion, short-term post-ACT (4–8 weeks) and long-term post-ACT (20–29 weeks) were used to assess CD8+ T cell characteristics.
Figure 2: CD8⁺ T-cell clonality in SOT recipients following adoptive immunotherapy. (A) Data represent the proportion of productive rearrangements when clones are grouped by frequency into small, medium, large, or hyperexpanded. (B) Data represent the productive clonality in SOT recipients before and after immunotherapy. (C) Data show a correlation between fold change in productive clonality long-term post-therapy and T-cell clonality prior to the commencement of adoptive cell therapy. Significance was determined using a two-tailed non-parametric Spearman’s correlation.
Figure 3: Changes in the clonotypic composition of the peripheral blood CD8\(^+\) T-cell repertoire following adoptive immunotherapy. Significant changes in the frequency of patient CD8\(^+\) T-cell clonotypes following adoptive cellular therapy were determined using the immunoSEQ platform. Significance was assessed in CDR3 sequences with a minimum of five reads. Significance was determined using a two-sided binomial test with Benjamini–Hochberg multiple comparisons’ correction, where \( \alpha = 0.01 \). (A) Representative analyses from three patients comparing the frequency of T cell clonotypes in pre-infusion and long-term post-therapy blood samples. Significantly expanded clonotypes post-therapy are shown in red.
Clonotypes that show a significant reduction post-therapy are shown in blue. (B) Data represent the number of clonotypes in each patient that displayed significant expansion post-therapy. (C) The frequencies of TRBV families detected in significantly expanded clonotypes from each patient, represented as a proportion of the total CD8+ T-cell population. (D) GLIPH analysis was performed to determine the relationship between expanded clonotypes. Data represent CDR3 motifs enriched after immunotherapy in three responding patients.
Figure 4: Characterisation of the clonotypic composition of cell therapy products. (A) The frequency of CD8+ T cells recognising CMV-encoded HLA-matched peptide epitopes was determined using a standard intracellular IFN-γ assay. Data represent IFN-γ-producing CD8+ T cells responding to individual peptide epitopes as a proportion of the response detected with the CMV peptide pool containing all peptide epitopes. (B) Circos plots showing the V and J gene pairings for T cell receptor β chain sequencing of therapy products. Ribbon thickness indicates number of pairings. Each colour represents an individual TRBV or TRBJ family.
**Figure 5: TRBV and CDR3 clonotypic overlap between ACT products.** (A) Heat map showing the productive frequency of TRBV gene usage in cell therapy products. (B) The immunoSEQ platform was used to determine overlap in CDR3 sequences between patient cell therapy products that shared HLA-restricted peptide epitope responses. Venn diagrams represent the overlap between patients with a HLA-A*01:01-restricted VTE-specific response, a HLA-A*02:01-restricted NLV-specific response, a HLA-B*07:02-restricted TPR-specific response and a HLA-C*06:02-restricted TRA-specific response.
Figure 6: Reconstitution of cell therapy-associated clonotypes following adoptive immunotherapy. The 20 most prevalent CDR3 sequences in the cell therapy product from each patient were determined. (A) Coloured slices in each pie chart represent the proportion of the top 20 clonotypes relative to productive clonotypes isolated from each cell therapy product. Grey slices represent CDR3 sequences that are not in the top 20 clonotypes. (B) The top 20 clonotypes from patient-specific cell therapy products were tracked over time in the corresponding patients. Colours match the CDR3 sequences in the pie charts in panel A.
Figure 7: Tracking the association between significant clonotypic expansions and presence in the cell therapy products. The significantly expanded clonotypes from each patient (as outlined in Figure 1) were assessed for their presence in the T-cell therapy products. Data represent the frequency of significantly expanded clonotypes associated with the T-cell therapy (T cell therapy), present pre-therapy but not detected in the cell therapy (present), or not present pre-therapy and not detected in the cell therapy (not present).
**Figure 8: CMV-specific T-cell frequency and phenotype following adoptive cell therapy.** The frequency and phenotype of CMV-specific T cells pre-therapy and at long-term follow-up were assessed using HLA-matched MHC multimer analysis and the expression of CD27, CD28, CD45RA, CD57, CCR7, and CD95. (A) Data represent the frequency of HLA-matched MHC multimer-specific T cells (left axis) overlaid with the frequency of CMV-specific IFN-γ-producing CD8+ T cells (right axis). (B) Concatenated files were prepared from patient samples at each time point and tSNE analysis used to establish phenotypic populations in CD8+ T cells, including naïve, central memory (CM1 and CM2) and effector memory (EM1, EM2, and EM3). The gating strategy for each population and fluorescence intensity for each surface marker are shown in Supplemental Figure 3. Left panels represent the CD8+ T-cell populations in four responding patients. Data in the middle and right panels are overlaid with the corresponding MHC multimer-specific population.
Figure 9: T cell phenotypic changes in SOT recipients following adoptive cell therapy.

Concatenated files were prepared from patients samples at each time point and tSNE analysis used to establish phenotypic populations in CD8⁺ T cells. (A) Data represent the proportion of memory CD8⁺ T cell populations in total CD8⁺ T cells following adoptive cell therapy. (B) T cell polyfunctionality was assessed following stimulation with the CMV peptide pool used to generate the cell therapy. Polyfunctional cells were defined as those T cells exhibiting two or more functions (typically IFN-γ⁺CD107a⁺TNF⁺⁻). Monofunctional cells were those capable of
producing only one function (typically IFN-γ\(^+\)). Data represent the proportion of polyfunctional or monofunctional CD8\(^+\) T cells.
**Table 1:** Clinical characteristics of patients included in the current study

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<th>Patient Code</th>
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<th>Donor/Recipient CMV Status</th>
<th>Immuno-suppression</th>
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AZA: Azathioprine; CSA: Cyclosporin; EVR: Everolimus; LEF: Leflunomide; MePRD: Methylprednisolone; MMF: Mycophenolate; PRD: Prednisolone; TAC: Tacrolimus.CDV: Cidofovir; FOS: Foscarnet; GCV: Ganciclovir; VGCV: Valganciclovir.

CR: Complete response; PR: Partial response; NR: No response.
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*Significantly expanded clonotypes